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**Assessment of metal, metalloid and radionuclide bioaccessibility from mussels to human consumers, using centrifugation and simulated digestion methods coupled with radiotracer techniques**

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ABSTRACT: The dietary bioaccessibility of 7 elements ( $^{241}\text{Am}$ , Cd, Co, Cs, Mn, Se and Zn) in the Mediterranean mussels *Mytilus galloprovincialis* (Lamarck, 1819) was assessed for human consumers. In this respect we assessed and compared the proportion of elements associated with the cellular cytosolic (“soluble”) fraction vs. the bioaccessible fraction derived, respectively, from (1) differential centrifugation method and (2) simulated digestion method. Comparisons were carried out on both raw and cooked mussels. Results showed that (1) the centrifugation method systematically underestimated (up to a factor 4) element bioaccessibility in raw mussels compared to the *in vitro* digestion method (e.g., 10 vs. 42% for  $^{241}\text{Am}$ ), and (2) the cooking process (5 min at 200°C) lead to concentrating the elements in mussel tissues (e.g., by a factor 2 for Zn) and reducing their bioaccessibility. Overall, the simulated *in vitro* digestion method appears as a powerful tool for seafood safety assessment and cooking could contribute in reducing substantially the global trace element intake from mussel tissues (up to 65% for Cd and Cs).

**Keywords:** Bioavailability; seafood; digestion simulation; differential centrifugation; seafood safety

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45 Funding sources that supported the work described in the manuscript were from IAEA-MEL  
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47

48 The present work was mainly carried out using *in vitro* techniques; however it also involved the  
49 use of marine bivalves. All experimental studies were conducted in accordance with national and  
50 institutional guidelines for the protection of animal welfare.

51

## 1. Introduction

Among seafood, bivalves generally display a high capacity in bioaccumulating metals, which suggests that the risk for their human consumers may not be negligible (e.g., Chouvelon et al., 2008; Metian et al., 2008). This risk depends primarily on the dietary bioavailable fraction of metal (viz., the fraction that is actually assimilated from the food and can reach the systemic circulation of an organism), that depends itself on the metal speciation within the seafood soft tissues. Before the 1990's, it was generally assumed that the bioavailable fraction was reliably assessed by the metal content of the cytosolic ("soluble") fraction in the cells. Later on, studies on the bioavailability of metals focusing on the "insoluble" fraction of the cells showed that the use of the cytosolic fraction alone underestimated the fraction of the metals that was bioavailable to the higher trophic levels. Nowadays, it is considered that the metals contained in both cytosolic and organelles fractions better reflect the bioavailable fraction (Wallace and Lopez, 1996; Wallace and Luoma, 2003). More recently, simulated digestion methodologies were developed to provide a more realistic assessment of the dietary bioavailable fraction of contaminants (Oomen et al., 2003; Versantvoort et al., 2005; Amiard et al., 2008).

The objective of the present study was to assess the dietary bioaccessible fraction (viz., the fraction resulting from the digestive process that can potentially be assimilated by the organism) of selected elements from mussels to human consumers, using two different methods, i.e., differential centrifugation and *in vitro* simulated digestion. Both methods were coupled with highly sensitive radiotracer techniques by using the corresponding  $\gamma$ -emitting radiotracers of the selected elements, i.e., four metals (Cd, Co, Cs, Mn, Zn), one metalloid (Se) and one artificial radionuclide ( $^{241}\text{Am}$ ). It is noteworthy that  $\gamma$ -emitting radiotracers of Co and Cs are also radionuclides commonly associated with nuclear industry wastes and fallout from nuclear

weapon testing. The present work also investigated the effect of cooking (raw vs. cooked mussels) on the metal content in the mussel soft tissues and on the resulting change in dietary bioaccessibility.

## 2. Material and Methods

Individuals of the Mediterranean mussel, *Mytilus galloprovincialis* (n = 32; 4-6 cm length), were collected along the coast of the Principality of Monaco and exposed for 3 weeks to the 7 selected radiotracers ( $^{54}\text{Mn}$ ,  $^{60}\text{Co}$ ,  $^{65}\text{Zn}$ ,  $^{75}\text{Se}$ ,  $^{109}\text{Cd}$ ,  $^{134}\text{Cs}$  and  $^{241}\text{Am}$ ) dissolved in natural 0.45- $\mu\text{m}$  filtered seawater (closed-circuit 70-l aquarium; salinity: 36 p.s.u.; temperature:  $21 \pm 0.5^\circ\text{C}$ ; pH:  $8.0 \pm 0.1$ ; light/dark cycle: 12h/12h). Activity of the radiotracers measured in seawater over the exposure duration was: 0.23 kBq  $^{54}\text{Mn l}^{-1}$ , 0.48 kBq  $^{60}\text{Co l}^{-1}$ , 0.58 kBq  $^{65}\text{Zn l}^{-1}$ , 0.31 kBq  $^{75}\text{Se l}^{-1}$ , 1 kBq  $^{109}\text{Cd l}^{-1}$ , 0.58 kBq  $^{134}\text{Cs l}^{-1}$  and 0.14 kBq  $^{241}\text{Am l}^{-1}$ . These radiotracer additions corresponded to stable metal concentrations of 1.1 pmol  $\text{Mn l}^{-1}$ , 0.6 pmol  $\text{Co l}^{-1}$ , 30 pmol  $\text{Zn l}^{-1}$ , 7 pmol  $\text{Se l}^{-1}$ , 0.3 pmol  $\text{Cd l}^{-1}$ , 11 pmol  $\text{Cs l}^{-1}$ , i.e., concentrations more than two orders of magnitude lower than the background concentrations in open seas (Ward, 2000). During this period, scallops were fed twice daily a phytoplankton diet (*Isochrysis galbana*;  $5 \cdot 10^4$  cells  $\text{ml}^{-1}$ ) and the seawater and spike were renewed every day for one week then every two days (Hédouin et al., 2006). After the exposure, mussels were placed in clean flowing seawater for 2 days in order to remove the radiotracers loosely bound to the mussels and to clear the gut contents (Metian et al., 2007).

A subsample of the mussels (n = 16) was then collected and kept raw whereas the remaining individuals (n = 16) were placed individually in 50-ml glass beakers, covered and heated for 5 min at  $200^\circ\text{C}$  on a hot plate. This temperature of cooking was selected in order to reflect a

99 intermediate temperature between relative low cooking temperatures (~100°C in boiling water)  
100 and high ones (250 to 350°C with oven or barbecue).

101 In order to determine the possible effect of cooking on the radiotracer contents in the mussels,  
102 tissues and fluids of 8 raw and 8 cooked mussels were radioanalyzed. Gamma-counting was  
103 carried out according to the method described in Rodriguez y Baena et al. (2006), using a high-  
104 resolution  $\gamma$ -spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors  
105 (EGNC 33-195-R, Canberra<sup>®</sup> and Eurysis<sup>®</sup>) connected to a multi-channel analyzer and a  
106 computer loaded with a spectra analysis software (Interwinner<sup>®</sup> 6).

107 For the remaining mussels (n = 8 for both cooked and raw individuals), the whole soft tissues  
108 were treated (1) by differential centrifugation according to the method described in Bustamante  
109 and Miramand (2005) to isolate the cellular cytosolic fraction by ultracentrifugation (28,000 G  
110 for 1 h, using a Sorvall RC28S ultracentrifuge) or (2) according to the *in vitro* simulated  
111 digestion method as described by Versantvoort et al. (2005). Briefly, this latter method consists  
112 of a three-step procedure which simulates quite closely the human digestive processes occurring  
113 in the mouth, stomach and small intestine (Versantvoort et al., 2005; Brandon et al., 2006;  
114 Amiard et al., 2008). The food matrix was first minced at 4°C and exposed to artificial saliva at  
115 pH 6.8 for 5 min. Artificial gastric juice at pH 1.3 was then added for 2 h and finally a mixture of  
116 artificial duodenal juice, bile and HCO<sub>3</sub> at pH 8.1–8.2 was added for a further 2 h. The incubation  
117 temperature was 37.2 ± 0.2°C. Chemicals and enzymes used are the same as described in  
118 Versantvoort et al. (2005) and Amiard et al. (2008) and were purchased from Sigma<sup>®</sup>. After the  
119 *in vitro* digestion, the resulting chyme was centrifuged at 1,572 G for 15 min at 37.2 ± 0.2°C.  
120 According to Versantvoort et al. (2005), the elements in the supernatant are representative of  
121 those occurring in the food (i.e. the mussels) and that are bioaccessible to humans (i.e. the

fraction that can be absorbed by the human gut enterocytes). Pellets and supernatants from both treatments (centrifugation and simulated digestion) were radioanalyzed in order to determine the activity of the 7 radiotracers.

### 3. Results

The overall results of the subcellular fractioning and simulated *in vitro* digestion methods showed that the bioaccessible fraction of the metals in mussels varied from 10% ( $^{241}\text{Am}$  in raw mussels) to 92% ( $^{134}\text{Cs}$  in raw mussels) (Table 1). However, for all the studied elements, the bioaccessible fraction determined using the *in vitro* digestion method was always higher (by a factor of up to 4) than the one assessed using the differential centrifugation method (Table 1).

Examination of Table 2 indicates that the cooking process resulted in concentrating systematically all elements but  $^{134}\text{Cs}$  in the mussel soft tissues (by 20 to 70%) and in releasing a significant amount of all elements but  $^{241}\text{Am}$  in the cooking juice.

It was also observed that although cooking increased the element concentration in the mussel flesh, the elements remaining in the cooked flesh were less bioaccessible than when occurring in raw tissues, in particular for Cd, Se, and Zn (Table 1).

### 4. Discussion

The results of our study show clearly that the bioaccessible fraction determined using the simulated *in vitro* digestion method was systematically higher (by a factor of up to 4) than when assessed using the differential centrifugation method (see Table 1). In particular, the latter method considerably underestimated the bioaccessibility of  $^{241}\text{Am}$ , Co, Mn, Se and Zn. This



observation was not that surprising as, from the theory, it was expected that the differential centrifugation method would provide a lower bioaccessibility estimate since it is only related to the cytosolic subcellular fraction (Wallace and Luoma, 2003). In contrast, by definition, the simulated *in vitro* digestion method would allow assessing the global metal fraction which is bioaccessible, regardless of the subcellular partitioning of the elements. By using a simplified empirical digestion simulation (acetic acid solution at pH 4), Bragigand et al. (2004) had already shown that some metals (Ag, Cd, Cu and Zn) could be bioavailable from the insoluble subcellular fraction of oyster cells. The *in vitro* digestion previously developed by Oomen et al. (2003) and Versantvoort et al. (2005) and used in the present study is a step forward to evaluate bioaccessibility of metals from seafood products by humans as it mimics quite closely the conditions occurring all along the human digestive tract (constant temperature, succession of enzymatic activities and pH, corresponding to each digestive step occurring from the mouth to the intestine).

The analysis of radiotracer content in raw and cooked mussels demonstrated that the cooking process resulted in concentrating by 20 to 70% most of the studied elements due to the loss of moisture as well as in releasing metals into the cooking juice. In particular, the cooking juice displayed metal concentrations higher by up to one order of magnitude than in the inter-valve fluid of raw mussels (see Table 2), corresponding to similar differences in terms of metal load (both cooking juice and inter-valve fluid were of similar volume).

Nevertheless, the comparison of our data with those previously published showed that a trend can hardly be generalized regarding the cooking effect on the metal content in seafood. Indeed, while some elements were found to concentrate in seafood after cooking (e.g. total and inorganic As in fish and molluscs; Devesa et al., 2001), other elements were not (e.g. Cd, Cu, Pb and Zn in the fish *Tilapia nilotica*; Atta et al., 1997). Furthermore, the element concentration in tissues after

cooking appears to depend on (1) the species considered (e.g.  $^{134}\text{Cs}$  is not concentrated in cooked mussel tissues whereas it is in fish tissues; present study and Burger et al., 2004, respectively), (2) the element considered and its chemical speciation (Devesa et al., 2001), and (3) the type of cooking (Ersoy et al., 2006). Although the aforementioned factors are generally reported as influencing the cooking effect, it has to be noted that the species-dependence factor could be partly due to the difference in tissue consideration when different species are investigated. Indeed, studies dealing with bivalves as ours generally consider the whole soft tissues as edible target, whereas fish related studies generally consider only the muscle tissues (fillet). Therefore, differences in the nature of the tissues (e.g., protein composition and moisture content) and in metal interactions with the cellular components in different tissues could lead to contrasting results. This is particularly true when storage organs such as liver / hepatopancreas or kidneys are considered. Indeed these organs are the main sites where detoxification processes take place, which usually result in an increase of excretion capacity or, more generally, in an increase in sequestration capacity and thus in different binding strength of the metals with cellular components (e.g., Metian et al., 2005).

Finally, although cooking resulted in an increase in element concentration in whole mussel flesh, it also appeared that the elements remaining in the cooked flesh were significantly less bioaccessible than those occurring in the raw tissues. This was particularly obvious for Cd, Se and Zn (see Table 1).

Considering the simulated *in vitro* digestion method and taking into account all the parameters (i.e., change in weight and in metal concentration of the cooked flesh, change in bioaccessible fraction), we have assessed that the metal intake for a consumer eating mussels would be reduced by 25% for Mn, 35% for Zn, 40% for Co, 50% for Se and 65% for Cd and  $^{134}\text{Cs}$  if the mussels are previously cooked and the cooking juice discarded before consumption. This decrease in

metal intake would be even more important if the bioaccessibility assessed via the subcellular fractioning method was considered (as the decrease in bioaccessibility between raw and cooked mussels was more marked). However, the information provided by the latter method is probably not directly comparable between raw and cooked mussels. Indeed, cooking of the flesh results in changes/damages of the cellular structure (e.g., protein agglutination), which will affect substantially the cytosol (both in its nature and occurrence) as well as the results of a centrifugation approach and their meaning. Hence the use of the centrifugation fractioning method is most probably relevant and informative only when the raw product is considered.

## 5. Conclusion

The simulated *in vitro* digestion method represents a powerful tool for the safety assessment of commercially important seafood products. Our study indicates that the bioaccessible fraction of metals in mussel soft tissues for human consumers depends on the metal chemical properties (e.g. distribution among cellular components and binding properties) and on the preparation of this seafood (in the present case, raw vs. cooked at 200°C for 5 min). The present work also showed that the cooking process generally concentrated the elements in mussel soft tissues, decreased their bioaccessibility for the consumers and released a significant part of the elements into the cooking juice. Results indicated that, providing the cooking juice is discarded, consumption of cooked mussels can contribute in reducing significantly the global intake of trace elements from seafood in humans (by as much as 65% for Cd and <sup>134</sup>Cs). Further studies are required on seafood containing norm-exceeding levels of trace elements in order to assess whether the risk for consumers could be decreased in cooked products down to levels allowing safe marketing. This

could be of particular interest in the case of Cd for which the maximum concentration allowed in marketed bivalves is low ( $1 \mu\text{g g}^{-1}$  wet wt; EC, 2006).

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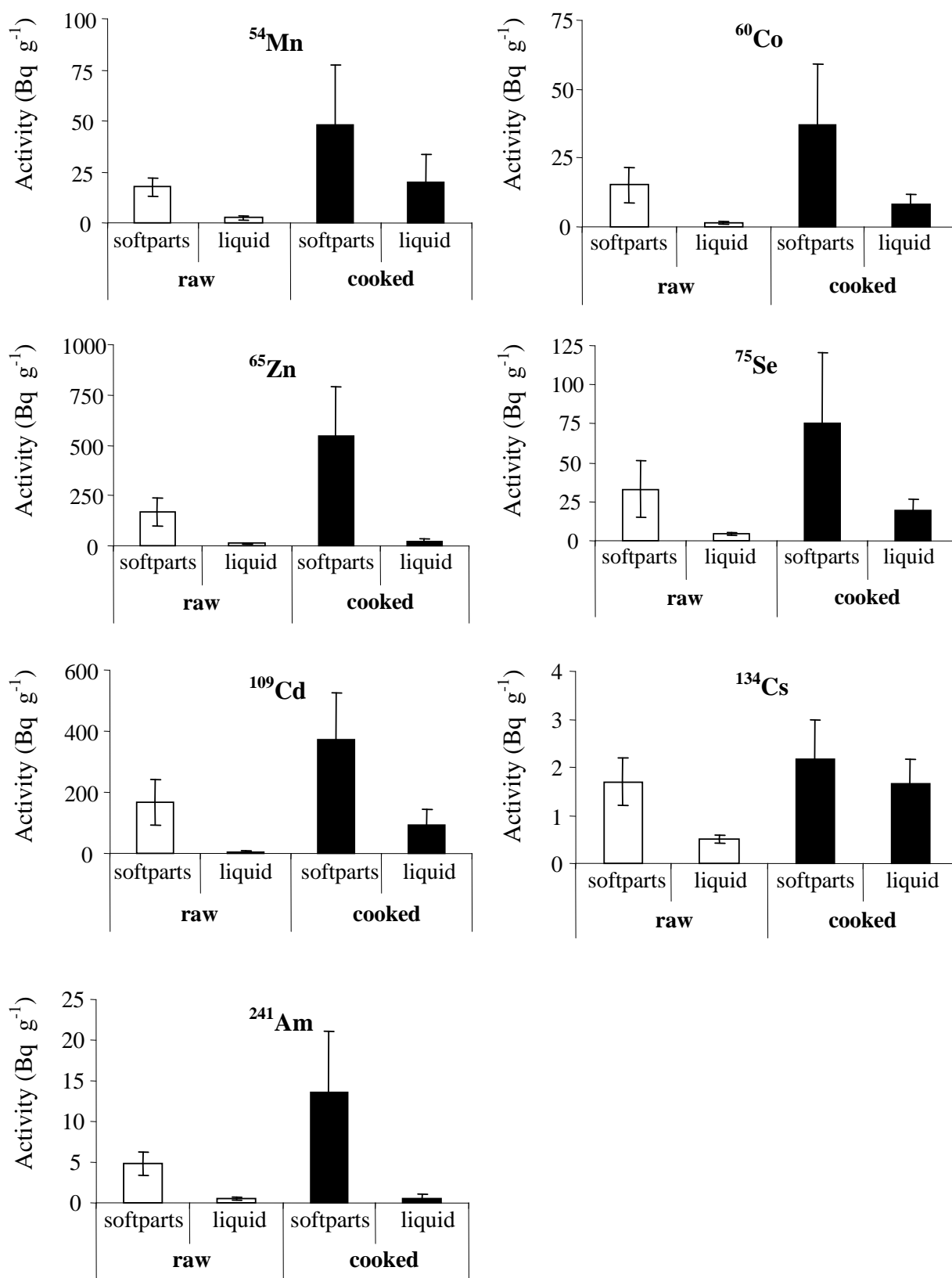
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**Figure 1. Repartition of radiotracer activities (Bq g<sup>-1</sup>) between tissues and liquid of the mussel *Mytilus galloprovincialis* before and after a cooking process.**



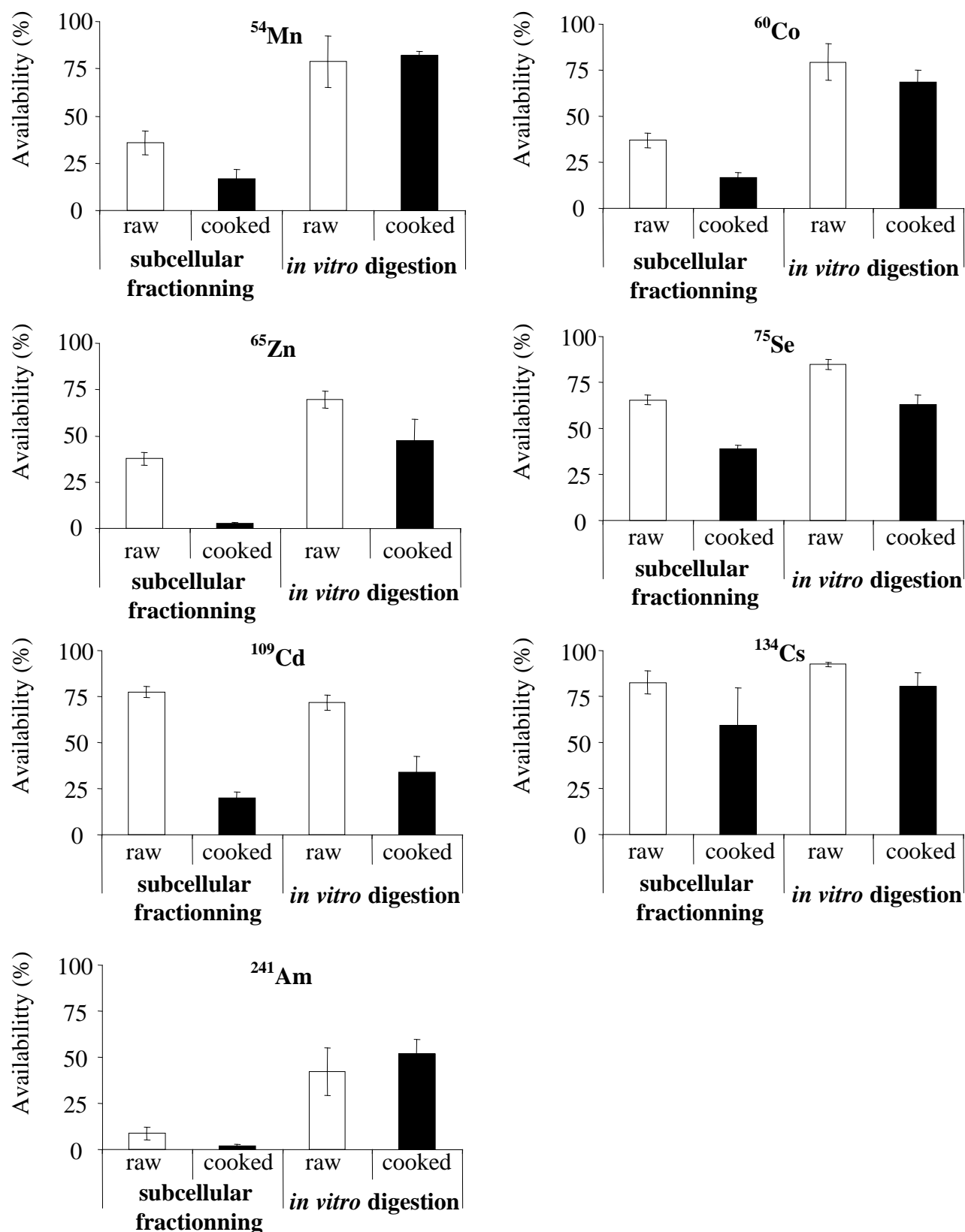


Figure 2. Radiotracer availabilities (% of initial activity in tissues) for mussel consumers following either subcellular or digestion methods (with or without the cooking process).

**Table 1.** Radiotracer bioaccessibility (%; mean  $\pm$  SD, n = 8) from mussel soft tissues (raw or cooked) to human consumers, assessed using either differential centrifugation fractioning method or simulated *in vitro* digestion method.

Mussel preparation	Method for assessing bioaccessibility	$^{54}\text{Mn}$	$^{60}\text{Co}$	$^{65}\text{Zn}$	$^{75}\text{Se}$	$^{109}\text{Cd}$	$^{134}\text{Cs}$	$^{241}\text{Am}$
Raw	fractioning	36 $\pm$ 6	37 $\pm$ 4	38 $\pm$ 4	65 $\pm$ 3	77 $\pm$ 3	82 $\pm$ 6	10 $\pm$ 3
	digestion	79 $\pm$ 13	79 $\pm$ 10	70 $\pm$ 5	85 $\pm$ 3	72 $\pm$ 4	92 $\pm$ 1	42 $\pm$ 13
Cooked	fractioning	17 $\pm$ 5	17 $\pm$ 3	3 $\pm$ 1	39 $\pm$ 2	20 $\pm$ 3	59 $\pm$ 21	2 $\pm$ 1
	digestion	82 $\pm$ 2	68 $\pm$ 7	47 $\pm$ 12	63 $\pm$ 5	34 $\pm$ 9	80 $\pm$ 8	52 $\pm$ 8

**Table 2.** Radiotracer activity (Bq g<sup>-1</sup> wet wt; mean ± SD, n = 8) in mussel soft tissues and fluid before and after cooking. Fluid: inter-valve fluid (in raw mussels) or cooking juice (in cooked mussels).

Mussel preparation	Compartment	Weight (g wet wt)	<sup>54</sup> Mn	<sup>60</sup> Co	<sup>65</sup> Zn	<sup>75</sup> Se	<sup>109</sup> Cd	<sup>134</sup> Cs	<sup>241</sup> Am
Raw	soft tissues	2.4±0.7	41±10	34±9	379±74	73±27	366±64	4±0.6	11±2
	fluid	5.4±1.9	13±7	7±4	60±26	23±5	30±13	3±0.7	3±2
Cooked	soft tissues	1.3±0.5	54±21	42±17	648±152	86±34	450±133	3±0.6	15±5
	fluid	4.1±1.3	75±49	32±13	74±17	74±22	322±87	6±1	2±2